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13. ABSTRACT ***(Maximum 200 Words)***

c-myb, along with A-*myb* and B-*myb,* belongs to the *myb* gene family which codes for nuclear proteins that bind DNA in a sequence-specific manner and function as regulators of transcription. There is a large body of evidence to suggest a role for c-*myb* in breast development and cancer. c-*myb* is highly expressed in all estrogen receptor positive breast tumors and cell lines. In addition, our *in situ* hybridization studies show that c-*myb* is expressed at high levels in ductal cells from breast tissues of virgin and pregnant mice. To address the role of c-*myb* in mammary development and cancer, we propose to create c-*myb* conditional knockout mice where the expression of this gene is interrupted specifically in the mammary gland using the Cre-*lox* system. We are in the process of generating chimeric mice by injecting our conditional c-myb deletion ES cells into blastocysts of C57 X CBA pseudopregnant mice. Founder mice will be identified and bred to homozygosity for carrying conditional c-myb deletion alleles and, additionally, to bear either a MMTV-cre or a WAP-cre transgene. The generation of breast-specific c-myb conditional knockout mice will afford us the opportunity to dissect the role of c-myb in normal breast development and cancer.

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Introduction

The development of breast cancer is a multistage process involving alterations in tumor suppressor genes and oncogenes. Overexpression of c-myb oncogene has been reported in human estrogen receptor positive (ER+) tumors and ER+ mammary carcinoma cell lines (1,2,3, also our unpublished data). In fact, cumulative data have shown c-myb to be one of the most frequently altered genes in breast cancer (1,4,5). In addition, recent data have shown a correlation between c-myb oncogene amplification and hereditary BRCA1 breast cancer (6). c-*myb,* along with A-*myb* and B-*myb,* belongs to the *myb* gene family which codes for nuclear proteins that bind DNA in a sequence-specific manner and function as regulators of transcription (7,8).

c-myb is predominantly expressed in hematopeoietic cells and its essential role for the proliferative potential of these cells has been well established (9). Homozygous null c-myb mutant mice die in utero due to defects in fetal hepatic hematopoiesis (10). However, the role of c-myb in breast development and breast cancer is beginning to emerge only recently. The first evidence that implicated a role for c-myb in breast tumors came from the observation that this gene is highly expressed in all estrogen receptor positive (ER+) breast tumors and mammary carcinoma cell lines (1,2,3, also our unpublished data). In addition, expression of a dominant negative mutant of the c-myb in ER+ breast carcinomas was found to result in their growth arrest and loss of tumorigenicity (our unpublished observations). To determine whether c-myb gene plays a role in breast development, we examined the pattern of expression of this gene in breast tissues derived from virgin, pregnant and lactacting mice. *In situ* hybridization studies show that c-myb is expressed at high levels in ductal cells derived from breast tissues of virgin and pregnant mice but is down-regulated in breast tissue of lactating mice. This observation combined with the observation that c-myb is highly expressed in ER+ breast tumor cells suggests that this gene might play a critical role in estrogen-mediated ductal cell proliferation.

Body

The main objective of my application is to study the effects of c-myb gene deletion on breast development using the embryonic stem (ES) cell technology and the Cre-*lox* system.

During my first grant year, I was unable to produce any type II conditional c-myb deletion ES clones but did learn the techniques for working with ES cells. This past year, I was able to isolate one type II ES clone RW4/38/33 from one of the recombinant ES cell clones that had been passed on to me. However, the karyotype of RW4/38/33 was abnormal. The other recombinant ES clones that I inherited either were karyotypically abnormal or could not yield type I systemic or II conditional c-myb deletion ES cells. So, I had to make new recombinant c-myb ES cell clones by electroporating the linearized targeting vector into RW4 ES cells, selecting with G418, and screening by Southern blotting with exon 9A probe after StuI digestion. After screening 300 clones, I did not get any true positive recombinant ES clones except for many false positive recombinant clones due to incomplete digestion by StuI restriction enzyme.

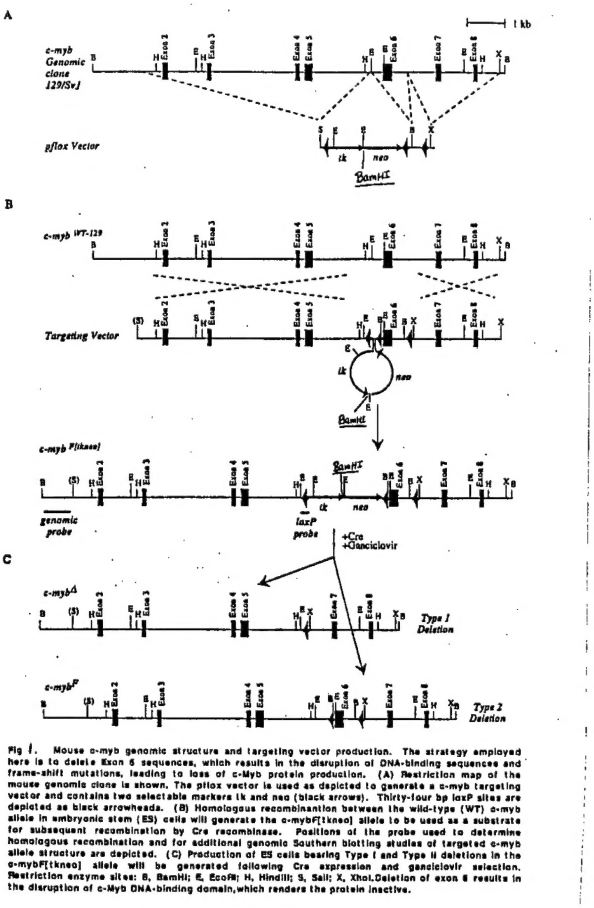

Fig 1. Mouse c-myb genomic structure and targeting vector production. The strategy employed here is to delete Exon 6 sequences, which results in the disruption of DNA-binding sequences and frame-shift mutations, leading to loss of c-Myb protein production. (A) Restriction map of the mouse genomic clone is shown. The pflox vector is used as depicted to generate a c-myb targeting vector and contains two selectable markers tk and neo (black arrows). Thirty-four bp loxP sites are depicted as black arrowheads. (B) Homologous recombination between the wild-type (WT) c-myb allele in embryonic stem (ES) cells will generate the c-myb^P[tkneo] allele to be used as a substrate for subsequent recombination by Cre recombinase. Positions of the probe used to determine homologous recombination and for additional genomic Southern blotting studies of targeted c-myb allele structure are depicted. (C) Production of ES cells bearing Type I and Type II deletions in the c-myb^P[tkneo] allele will be generated following Cre expression and ganciclovir selection. Restriction enzyme sites: B, BamHI; E, EcoRI; H, HindIII; S, SalI; X, XhoI.Deletion of exon 6 results in the disruption of c-Myb DNA-binding domain,which renders the protein inactive.

Figure 1: The modified c-myb targeting construct contains an extra BamHI restriction site between the tk and Neo genes.

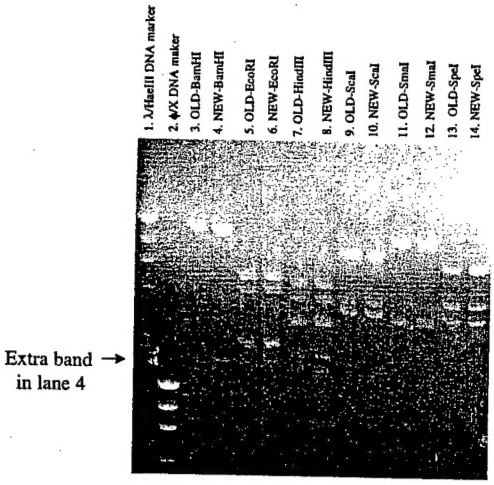

Figure 2: Agarose gel comparing the restriction banding patterns between the old and new targeting vector. Notice the new vector has an extra band as indicated by the arrow mark.

With the help of another lab member, I decided to modify the original targeting vector by

simply inserting a BamHI linker in the region between the thymidine kinase gene and the neomycin resistant gene (Fig 1). As shown in figure 2, the restriction band patterns on the agarose gels were the same for the new and old targeting vectors for all enzymes examined except BamHI where the new vector yielded an extra band as expected. This modified targeting vector enables efficient screening of c-myb recombinant clones by digestion with BamHI and using a probe upstream of exon2.

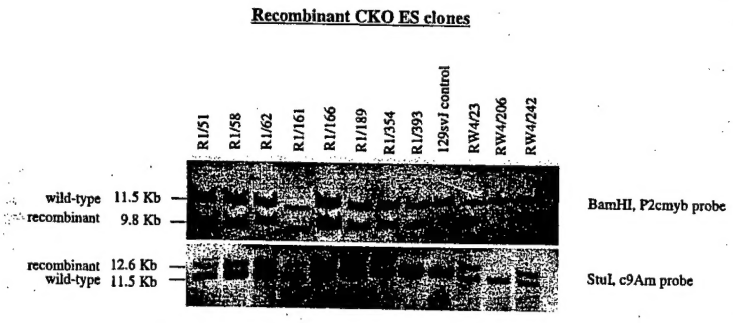

Figure 3: Southern blot analysis of the c-myb recombinant clones.

As shown on figure 3, when I introduced the new vector into both R1 and RW4 ES cells and after screening 900 clones, I was able to isolate 9 bona fide positive recombinant clones. I carried out cell spreading experiments to determine which of the 9 recombinant clones have normal chromosome number. R1/51, R1/62, R1/354, RW4/23, RW4/242, R1/58 and R1/161 have the normal murine 40 chromosomes and the first 5 recombinant clones were sent for karyotyping analysis by Dr. Joseph Testa of Fox Chase Cancer Center, Philadelphia, PA. Results showed that only R1/51, R1/62 and RW4/23 have normal karyotype. Various concentrations of P1 bacterial recombinase cre were electroporated into these three normal ES clones. As shown on figure 3, a total of 6 type II conditional c-myb ES deletions were isolated, 3 from R1/51 and 3 from RW4/23 recombinant c-myb clones. Two type II ES clones from each of the two first transfectants were sent for karyotyping analysis by Dr Chih-Lin Hsieh of the University of Southern California and all 4 type II deletion ES clones, R1/51.29/5, R1/51.18/82, RW4/23/47 and RW4/23/62, have normal karyotype. I am in the process of generating chimeric mice by having two of the four type II conditional c-myb deletion ES clonal cells, R1/51.18/82 and RW4/23/47, microinjected into blastocysts of C57B/L6 X CBA pseudopregnant mice by Dr.

Thom Saunders of University of Michigan Transgenic Core Facility. Once I get chimeric mice, founder mice will be identified and bred to carry two copies of the conditional c-myb deletion alleles and also to bear either a MMTV-cre or a WAP-cre transgene.

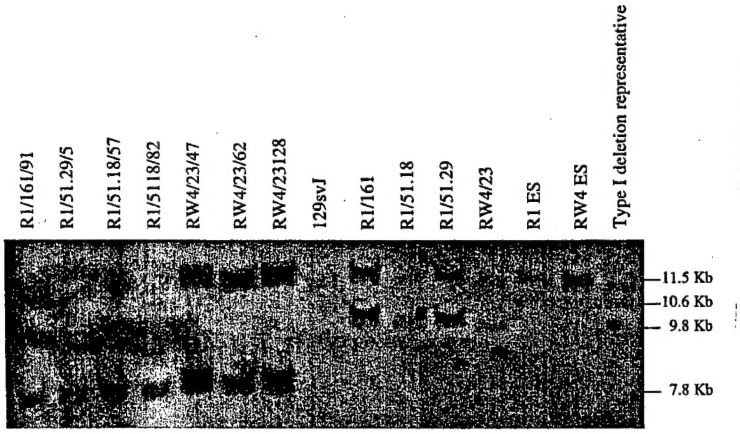

Figure 4: Southern blot analysis showing the type II conditional c-myb deletion clones. 11.5 Kb-wt, 10.6 Kb-type I systemic c-myb deletion, 9.8 Kb-recombinant and 7.8 Kb-type II conditional c-myb deletion.

Key Research Accomplishments

- Construction of an improved c-myb targeting vector for efficient screening of recombinant clones.
- Isolation of at least 3 normal recombinant c-myb ES clones.
- Production of at least 4 normal type II conditional c-myb deletion ES clones.

Reportable Outcomes

None

Conclusions

We have isolated at least 3 normal recombinant c-myb ES clones using a modified c-myb targeting vector that we have constructed. After introduction of the P1 bacterial recombinase cre into these recombinant clones, we have obtained at least 4 normal type II conditional c-myb deletion ES clones which are currently being microinjected to produce chimera mice. We believe the generation of breast-specific c-myb conditional knockout mice will provide an invaluable model for dissecting the role of c-myb in normal development as well as gain insight

into the role of its aberrant expression in breast cancer. Furthermore, we believe that a detailed molecular understanding of how c-myb contributes to tumor progression is of major importance for future therapy.

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